PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

PCT

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43*bis*.1)

Date of mailing
(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION
See paragraph 2 below

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB2004/001167	18.03.2004	24.03.2003

International Patent Classification (IPC) or both national classification and IPC
G01N33/58, G01N33/68, C07K1/18

Applicant
XZILLION GMBH & CO. KG

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☒ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☒ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA"). However, this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notifed the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of three months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/GB2004/001167

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of the international application in the language in which it was field, unless otherwise indicated under this item.

   ☐ This opinion has been established on the basis of a translation from the original language into the following language , which is the language of a translation furnished for the purposes of international search (under Rules 12.3 and 23.1(b)).

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

   ☐ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☐ in written format

   ☐ in computer readable form

   c. time of filing/furnishing:

   ☐ contained in the international application as filed.

   ☐ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application,

☒ claims Nos. 1-13, 15-39 (partially) and 14 (entirely)

because:

☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination *(specify)*:

☐ the description, claims or drawings *(indicate particular elements below)* or said claims Nos. are so unclear that no meaningful opinion could be formed *(specify)*:

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the whole application or for said claims Nos. 1-13, 15-39 (partially) and 14 (entirely)

☐ the nucleotide and/or amino acid sequence listing does not comply with the standard provided for in Annex C of the Administrative Instructions in that:

the written form	☐	has not been furnished
	☐	does not comply with the standard
the computer readable form	☐	has not been furnished
	☐	does not comply with the standard

☐ the tables related to the nucleotide and/or amino acid sequence listing, if in computer readable form only, do not comply with the technical requirements provided for in Annex C-*bis* of the Administrative Instructions.

☐ See separate sheet for further details

Box No. IV Lack of unity of invention

1. ☒ In response to the invitation (Form PCT/ISA/206) to pay additional fees, the applicant has:

   ☐ paid additional fees.

   ☐ paid additional fees under protest.

   ☒ not paid additional fees.

2. ☐ This Authority found that the requirement of unity of invention is not complied with and chose not to invite the applicant to pay additional fees.

3. This Authority considers that the requirement of unity of invention in accordance with Rule 13.1, 13.2 and 13.3 is

   ☐ complied with

   ☒ not complied with for the following reasons:

   see separate sheet

4. Consequently, this report has been established in respect of the following parts of the international application:

   ☐ all parts.

   ☒ the parts relating to claims Nos. 1-13, 15-39 (all partially)

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-13,15-39 (all partially)
	No:	Claims	-
Inventive step (IS)	Yes:	Claims	-
	No:	Claims	1-13,15-39 (all partially)
Industrial applicability (IA)	Yes:	Claims	1-39
	No:	Claims	-

2. Citations and explanations

   see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY (SEPARATE SHEET)

International application No.

PCT/GB2004/001167

Re Item III.

N. The International Search Authority has found that the present application relates to separate inventions or groups of inventions. As no additional search fee has been paid, only the subject-matter relating to the invention first mentioned in the claims has been searched (see point U.4[a] below). The present application will be limited accordingly (Rule 66.1(e) PCT).

Re Item IV.

U. UNITY (Rule 13.1 PCT).

U.1 The present application concerns tagging agents for mass spectrometry.

U.2 The common concept, which would link the claimed subject-matter together, is the idea of attaching tertiary amine tags to the free amino groups of the analyte molecules.

U.2[a] This idea is not novel because the prior art discloses the N-terminal derivatization of peptides with tertiary amines for the purpose of determining the amino acid sequence by mass spectrometry (see: Haralambidou et Al., page 684, the second and the third paragraphs, and the second paragraph on page 696; Day et Al., abstract and figures 1-9).

U.2[b] Hence, this idea cannot be considered a single general inventive concept according to Rule 13.1 PCT, and a lack of unity "a posteriori" is indicated.

U.3 Apart from the common concept identified above, the claims concern:
(i) different reagents and methods for attaching the mass tags to the free amines of the analyte molecules, each of them comprising/involving a specific amino-reactive functionality (see claims 2, 5, 11, 23, 24 and 30), (ii) different structures of the tertiary amine tag (see claims 3, 6-10 and 23-29), (iii) different structures of the linker between the tertiary amino group and the amino-reactive functionality of the tagging reagents (see claims 4, 12, 13, 23, 31 and 32), (iv) ensembles of different tagging agents for the differential labelling of a plurality of analytes, and the use of such a mass tagging procedure in combination with (v) the cleavage of a peptidic analyte molecule or (vi) the chromatographic purification of the analyte.

U.3[a] In view of the prior art, there is no single technical relationship among these different embodiments of the claimed subject-matter involving one or more of the technical features to which an inventive step could be addressed (Rule 13.2 PCT). Hence, they are considered to relate to separate inventions or groups of inventions as defined below.

U.4[a] Subject 1: claims 1-13, 15-39 (all partially).
Mass tag reagents having esters of carbonic acids as the amino-reactive functionalities, their kits and uses for the characterization of molecules by mass spectroscopy and/or for molecule purification. Claims 1-39 are partially to be considered within this group in so far as the reactive functionalities of the mass tag reagents are esters of carbonic acids.
(It is noted that active esters of carbonic acids are different from the N-hydroxysuccinimide esters, which are disclosed in the examples and referred to in the following groups, because these are esters of carboxylic acids rather than of carbonic acids.)

U.4[b] Subjects 2-18: claims 1-13, 15-39 and eventually claim 14 (all partially).
Mass tag reagents having the amino-reactive functionalities selected from the list below, their kits and uses for the characterization of molecules by mass spectroscopy and/or for molecule purification. Each class of amino-reactive functionalities listed below relates to a separate group of inventions, and claims 1-39 are partially to be considered within this group depending on the amino-reactive functionalities of the mass tag reagents. The separate classes of amino-reactive functionalities are: alkenyl sulphones, haloalkanes, maleimides, isocyanates, isothiocyanates, ketones, aldehydes, sulphonyl-halides, carboxylic-halides, anhydride esters, alkenes, N-hydroxysuccinimide esters, hydroxybenzotriazole esters, hydroxyazabenzotriazole esters, nitrophenyl esters, trichlorophenyl esters, pentafluorophenyl esters.

U.4[c] Subjects 19-21: claims 1-39 (all partially, one or more of claims 6-10, 14, 22 and 25-29 are to be entirely excluded).
Mass tag reagents, their kits and uses for the characterization of molecules by mass spectroscopy and/or for molecule purification. A mass tag reagent according to these groups of inventions comprises a tertiary amine with two alkyl substituents that are: (i) separated, or (ii) linked together as to form a cyclic group with the nitrogen atom, or (iii) linked together as to form a cyclic group with the

nitrogen atom and a further heteroatom. Each of these tertiary amine structures relates to a separate group of inventions, and claims 1-39 are partially to be considered within this group, or eventually excluded in their entirety, depending on the structure of the tertiary amine group.

U.4[d] Subjects 22 and 23: claims 1-39 (all partially, eventually excluding claims 14 and 22 in their entirety).
Mass tag reagents, their kits and uses for the characterization of molecules by mass spectroscopy and/or for molecule purification. A mass tag reagent according to these groups of inventions comprises an alkylene or a phenylene linker between the tertiary amine and the amino-reactive functionality. The alkylene and the phenylene linkers relate to two separate groups of inventions. Claims 1-39 are partially to be considered within any of these groups, and claims 14 and 22 are eventually to be excluded in their entirety, depending on the structure of the linker.

U.4[e] Subject 24: claims 1-18 (partially) and claim 19.
Method for the characterization of proteins and polypeptides by mass spectroscopy involving labelling the whole analyte and its fragments, which have been generated by means of a cleavage reagent, with mass tag reagents comprising a tertiary amine and an amino-reactive functionality. Claims 1-18 are partially to be considered within this group in so far as they only relate to the method of claim 19.

U.4[f] Subject 25: claims 1-20 (partially), 21, 22, 36 and 37.
Arrays of different mass tag reagents and method for the characterization of a plurality of molecules by mass spectroscopy involving labelling each molecule with a different mass tag reagent. Claims 1-20 are partially to be considered within this group in so far as they only concern a plurality of different mass tag reagents.

U.4[g] Subject 26: claims 38 and 39.
Kits for the purification of labelled analyte molecules comprising a mass tag reagent and a cation exchange resin.

Re Item V.

1. DOCUMENTS.

Reference is made to the following documents:

D1: Haralambidou E. & Day R. A., *Organic Mass Spectrometry* (1975) Vol. 10, Pages 683-697;
D2: Day R. A. et Al., *The Journal of Organic Chemistry* (1973) Vol. 38, No. 4, Pages 782-788;
D3: US 2003/0044864 A;
D4: WO 02/066988 A;
D5: US 5181698;
D6: Lewis M. R. et Al., *Bioconjugate Chemistry* (1994) Vol. 5, No. 6, Pages 565-576.

1.1 D1 and D2 disclose peptide N-terminal tagging agents for the determination of the peptide sequence by Mass Spectrometry. In particular, the tagging agents disclosed in D1 are dimethylaminobenzaldehyde and dimethylaminobenzoate succinimidyl ester (see: abstract; page 684, second and third paragraphs; figures 1-3; page 696, second paragraph), and the tagging agents of D2 are dimethylaminobenzaldehyde, dimethylaminonaphthaldehyde and dimethylaminonaphthalenesulfone (see: abstract; figures 1-9; table I).

1.3 D3 discloses mass spectrometric methods for the identification of one or more proteins by differentially labelling the peptide fragments from the enzymatic digestion of the proteins (see claims 1 and 50). In the preferred embodiments, the labels bind to the amino groups of the proteins by means of reactive functionalities like succinimide-activated carboxylic groups, isothiocyanates and isocyanates (see claims 11, and 59). Preferably, the labels have the same chemical structure, very similar properties, but different isotope composition (see claims 17, 51, 52). The specific labelling agents disclosed in D3 comprise also affinity ligands (see claim 53).

1.4 D4 discloses reagents and methods for the characterization of phosphorylated proteins (see abstract and claim 1). According to the procedure disclosed in D4, one or more proteins are labelled for mass spectrometric analysis, and the labelling method eventually involves different mass isotopes for differentially labelling the analytes (see: page 14, lines 3-7; claims 4 and 9). In some embodiments, the labelling agents comprise also ligands for the isolation of the labelled protein (see paragraph joining pages 16 and 17).

1.4[a] In particular, D4 indicates that the presence of a tertiary amine in the labelling agent improves ionization of the analyte (see page 15, lines 25-32).

1.5 D5 discloses the use of activated carbonate esters for attaching a desired chemical moiety to a free amino group of a protein (see column 2, lines 39-41, and figure 2).

1.6 D6 discloses the mass spectrometric characterization of a cytochrome "c" conjugate consisting of the protein, which is labelled with a chelating agent for radioactive metals comprising trialkylamino groups (see: abstract and page 569, left-hand column, lines 18-21). This protein conjugate is obtained by reacting the free amino groups of the protein with the N-hydroxysulfosuccinimidyl carboxylate ester of the labelling agent (see scheme 1).

1.6[a] In addition, D6 reports about the activation of protein labelling reagents by isothiocyanate (see page 566, left-hand column, fourth paragraph).

2. INDUSTRIAL APPLICABILITY (Art. 33(4) PCT).

2.1 Claims 1-39 relates to analytical methods based on mass spectrometry and labelling reagents useful in these methods. The labelling agents can be synthetized on industrial scale and used, together with the claimed methods, in the chemical and pharmaceutical industry. Hence, they are to be considered industrially applicable according to article 33(4) PCT.

3. NOVELTY (Art. 33(2) PCT).

3.1 In so far as it only concerns "Subject 1" (see point U.4[a] above), the method of independent claim 1 and the mass tag reagent of independent claims 23 are novel over the available prior art. None of the cited documents discloses compounds comprising a tertiary amine and a carbonic acid ester reactive functionality, nor mass labels with corresponding structural features.

3.1[a] For example, the peptide tagging reagents of D1 and D2 differ in that they comprise an activated carboxylic acid ester, an aldehyde, or a sulfone functionality for peptide binding (see point 1.1 above). With respect to that, it is noted that the claimed active esters of carbonic acids are different from the carboxylic acid esters disclosed in the prior art (see also point 1.6 above).

3.2 The subject-matter of dependent claims 2-13, 15-22, 24-35 and claims 36-39 is also novel because these claims specifically relates to the novel method and the novel reagent of claims 1 and 23.

4. INVENTIVE STEP (Art. 33(3) PCT).

4.1 Documents D1 and D2 are considered to represent the relevant state of the art because they discloses tagging agents, which contain tertiary amino groups and bind to peptide N-termini, for the determination of peptide sequences by Mass Spectrometry (see point 1.1 above).

4.1[a] The subject-matter of independent claims 1 and 23 differs in the reactive functionality binding to the free amino groups of the analyte molecules, said functionality being selected from active esters of carbonic acids, rather than from activated N-hydroxysuccinimidyl carboxylate esters, aldehydes, or sulfones.

4.2 The problem can therefore be regarded as the provision of an alternative reactive functionality for attaching a tagging agent with a tertiary amino group for mass spectrometry to a free amino group of the analyte molecule.

4.3 The solution proposed, i.e. the use of an active carbonate ester, does not involves any inventive step because it is to be considered a selection among possibilities within the skilled person customary practice that does not lead to any unexpected

effect or property.

4.3[a] The use of active carbonate esters for the purpose of attaching a desired chemical moiety to the free amino groups of a target molecule is known to the skilled person (see for example point 1.5 above). Hence, the skilled person would have considered to modify the tagging reagents of D1 and D2 (e.g. starting from the aldehyde functionalities to the active carbonate esters of the corresponding hydroxyl moieties) in order to solve the problem posed, thereby obtaining methods and mass tagging reagents as claimed. The fact that in the prior art there is no concern about the particular structure of the linkage between a mass tag and the analyte molecule represents an incentive for the skilled person to try any of the known alternative amino-reactive functionalities (see for example the fact that the preferred tagging agents of D1 and D2 have different reactive functionalities; see also claim 59 of D3, and points 1.6 and 1.6[a] above).

4.3[b] The present application does not provide any example of mass tagging reagent having active esters of carbonic acids as the amino-reactive functionalities, nor reports on the properties of such a mass tagging reagent. As no unexpected effects or properties are apparently achieved by means of these amino-reactive functionalities, the subject-matter of claims 1 and 23 cannot be considered as involving any inventive step.

4.4 Dependent claims 2-13, 15-22, 24-35 and claims 36-39 do not contain any feature which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of inventive step, given the disclosure of the prior art (see points 1.1, 1.3 and 1.4 above).

4.4[a] In particular, D3 and D4 suggest the use of different isotopically labelled tagging agents for the analysis of a plurality of analytes and the use of chromatographic techniques, among which ion-exchange chromatography could be expected to be suitable in view of the basicity of the tertiary amine tags, for the purification/isolation of the labelled analytes.

Re Item VIII.

5. CLARITY and SUPPORT (Art. 6 and 5 PCT).

5.1 Claim 1 does not meet the requirements of Article 6 PCT in that the matter for which protection is sought is not clearly defined. The claim attempts to define the subject-matter in terms of the result to be achieved, i.e. the ability of the reactive functionality to react with an amino group, without providing the technical features necessary for achieving this result. Such a definition is different from the definition of the corresponding reaction as a procedural step of the claimed method.

5.1[a] Moreover, the application provides support only for one class of reactive functionalities, namely for N-hydroxysuccinimide esters (see the examples).

5.2 Dependent claim 7 is unclear when referring to claim 6 because these claims define two sets of variables, which do not co-exist in any of the formulae of claim 5. Hence, claims 7 and 6 relates to two separate and incompatible embodiments and should not refer one another.

5.3 The term "derivative" used in claim 18 is vague and unclear and leaves the reader in doubt as to the meaning of the technical features to which it refers, thereby rendering the definition of the subject-matter of said claim unclear.

5.4 The abbreviation "DMG" is used in claim 22 without having been defined. This leaves the reader in doubt as to the meaning of said abbreviation and renders the definition of the subject-matter of said claim unclear.